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(57) Abstract

The present invention relates to oligonucleotides with RNA cleavage activity called ribozymes having sequence (I) in which ---X and Y--- are target specific RNA recognition sequences, A, C, G and U are deoxynucleotides and at least one a and/or g is a 2'-substituted adenosine or guanosine derivative respectively, P is either a) A-U or b) C-G and, W is either i) a nucleotide loop sequence or ii) dial bridges connected with phosphodiester or substituted neutral phosphotriester derivative linkages.

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Oligonucleotides with RNA Cleavage Activity

The present invention relates to oligonucleotides with RNA cleavage activity.

RNA is known to have endoribonuclease activity. Catalytic RNA molecules called ribozymes have been used to bind to target RNA molecules and catalyse their cleavage, thus blocking the activity of the target RNA. The so-called hammerhead ribozymes have been widely studied. The recognition site and catalytic site of these oligoribonucleotides are well characterised and ribozymes containing a recognition sequence specific for any desired target RNA which contains a specified triplet can be constructed. These compounds can therefore be considered as potential therapeutic agents with possibly higher biological activity than the simple antisense oligonucleotides[1].

The structure of a typical hammerhead ribozyme is shown in Figure 1. A G C and U are all ribonucleotides.

Experiments show that externally supplied ribozymes produce only a transient effect. The catalytic effect is destroyed once the ribozyme has been degraded. The accessibility of the target site also limits ribozyme cleavage activity. Protein bound at the cleavage site has been shown to block ribozyme activity. Further, ribozyme catalytic efficiency has been shown to depend on whether the ribozyme expression occurs in the same cell compartment as that occupied by the target DNA. The hammerhead ribozyme has been shown to have no effect in prokaryotic cells when the ribozyme and target RNA were generated from different genes, whereas it can function at a 1:1 ribozyme:target ratio if co-localization in the same cell is maintained. Studies[1] have shown that a high (1000:1) ribozyme:substrate ratio is needed for inhibition *in vivo* in eukaryotic cells. This suggests that the catalytic potential of the ribozyme is not being achieved.

Eckstein *et al* introduced 2'-amino or 2'-F substituents into the pyrimidine positions of the hammerhead ribozyme [2][3]. The

cleavage positions of the ribozymes in cellular extracts were not determined but the all-pyrimidine substitutions together with phosphorothiolate substitutions at the 3' terminus gave compounds with a markedly increased stability. The influence of these chemical modifications on the catalytic activity of the ribozymes was negligible.

Sproat *et al*[4] found that ribozymes containing 2'-O-Allyl substituents in all but six positions of the catalytic core are resistant to nuclease attack to some extent and retain their catalytic activity.

Cedergreen *et al*[5] found that mixed DNA / RNA oligomers with 4-7 ribopositions are active in cleaving substrate RNA. These oligomers are three orders of magnitude more stable than the all-RNA ribozymes in incubation with RNase A and yeast extract.

Recent results have suggested that the ribonucleotide backbone is not a strong requirement for catalytic cleavage of mRNA[4]. When the 2'-OH groups in the hammerhead structure were systematically replaced with H residues it was observed that the presence of only four 2'-OH groups at defined positions in the catalytic core (the core being nucleotides 3 to 15.1 shown in Figure 1) were necessary to show catalytic activity. Referring to Figure 1, these positions are G^5, G^8, A^9 and $A^{15.1}$. If the A^6, A^{14} and one nucleotide in helix III (possibly $C^{15.1}$) were also left unmodified, catalytic activity was found to reach 1/10th of the all-2'-OH RNA ribozyme.

If the substituents were 2'-O-Allyl groups the essential 2'-OH groups needed for catalytic activity were found to be U^4, G^5, A6, G^8, G^{12}, and $A^{15.1}$. Those shown in bold are positions identical to the DNA analogue. The kcat/Km for this ribozyme was only 5 times lower than for the all 2'-OH ribozyme.

Other modifications of the 2'-OH groups in the catalytic core of the hammerhead ribozyme have been reported. Williams *et al*[8] found only a 15-fold reduction in catalytic activity if the 2'-OH groups at G^5 and G^8 are replaced with 2'-NH_2 and the 2'-OH

group at G^{12} is replaced with a 2'-F group. Olsen *et al*[9] noted only a small decrease in catalytic activity if the 2'-OH group at one of the following adenosines : A^6, A^9 or $A^{15.1}$, A^{14}, A^{13} is replaced by 2'-F. Replacement of more 2'-OH-s, however, is not allowed with this substituent.

Pyle *et al*[6] studied the *Tetrahymena* ribozyme and showed that the essential -OH groups are important for stabilising base-backbone tertiary interactions by hydrogen bonding. It is not clear, however, whether this is the case, or whether the 2'-OH groups are important for the direct binding of the catalytically important metal ion. Perreault *et al*[7] suggest that the 2'-OH groups of A^9 and G^5 are directly involved in Mg^{2+} binding.

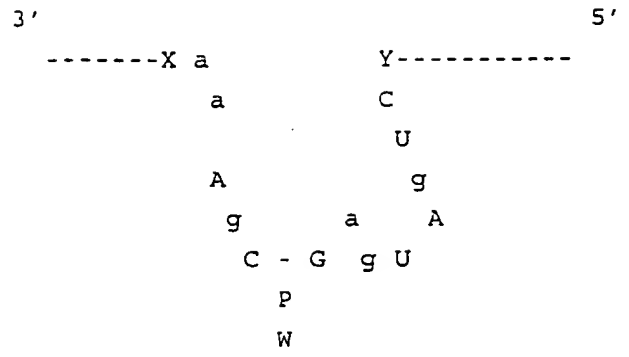
In a recent model McLaughlin *et al*[10] proposed a role for the 2'-OH as of G^5 or G^8. They interact with H_2O molecules bound in the first co-ordination sphere of the Mg^{2+} cofactor.

In an effort to determine the minimal sequence requirements for ribozyme activity Jennings *et al*[11] observed that the catalytic activity of a hammerhead ribozyme in which the loop of nucleotides 10.1 to 11.1 (see Figure 1) is replaced by four deoxyuridines decreases only by a factor 3 when compared with the original structure. This modified structure has been termed the "miniribozyme structure".

For exogenous application of preformed synthetic ribozymes several important problems can be identified. It is desirable to:

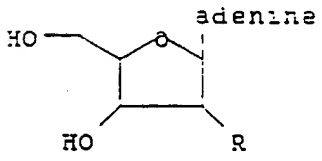
1. increase stability against RNase degradation
2. increase stability against exonuclease degradation and
3. facilitate cellular uptake.

The present invention provides in a first aspect oligonucleotides having the following sequence

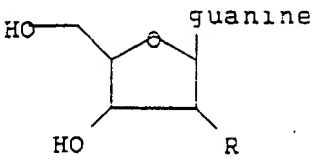


in which

----X and Y---- are target specific RNA recognition sequences
A is deoxyadenosine
C is deoxycytidine
G is deoxyguanosine
U is deoxyuridine
a is 2' substituted deoxyadenosine according to formula Ia below, or a 2'substituted arabinofuranosyl adenosine according to formula Ic below, or adenosine g is 2' substituted deoxyguanosine according to formula Ib below or a 2' substituted arabinofuranosyl guanosine according to formula Id below, or guanosine

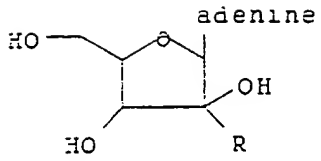


Ia

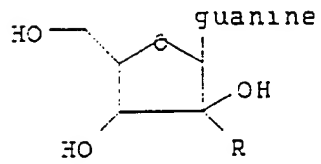


Ib

5



Ic



Id

in which R is selected from
$-CF_2H$; -COOH ; $-CONH_2$; $-NHCOR_1$; - SH ; and combinations thereof; R_1 being -H, -C(O)OH, or $-CH_2$-C(O)OH
and wherein at least one of the groups a or g of the oligonucleotide is other than adenosine or guanosine respectively;
and in which

P is either a) A-U or b) C-G

W is either i) nucleotide loop sequence IIa if P is A-U;

3′ 5′
-G-G-A-G-C-C-C-C- IIa

or nucleotide loop sequence IIb if P is C-G

3′ 5′
-C-C-G-U-U-A-G-G- IIb

or ii) diol bridges III connected with phosphodiester or substituted neutral phosphotriester derivative linkages

m(Z) III

in which each diol bridge (Z) is of formula IV

$$- \quad [\quad O - (CH_2)_n - O \quad] - \qquad IV$$

n is 1-10

and m diol bridges are connected together, m being 2-10.

There is no requirement that the length of the diol be the same in each diol bridge; n may be independently selected for each bridge from 1 to 10. Preferably n is 3 in each bridge and there are four bridges; m is 4.

X and Y are target specific recognition sequences made up of any deoxyribonucleosides N depending on the target RNA sequence. X and Y may be of the same or different length. There is no need for the molecule to be symmetrical. Each may be 4 to 25 nucleotides long, preferably 6 to 20 nucleotides. If X and Y are too short the oligonucleotide looses its specificity.

X or Y may include stabilising modifications. For example, two or three natural 3'-5' phosphodiester linkages at at least the 3' end of X may be modified in an attempt to protect the oligonucleotide from attack by 3'-exonucleases. Particularly, 3'-5' phosphodiester linkage may be replaced by phosphorothiate linkages such as thiophosphodiester linkages.

The 2'-R substituent is a non-nucleophylic group which is isoteric and isopolar with the replaced -OH group. The substituent has both H-bond donor and acceptor abilities. A 2'-COOH substituent, which can chelate with Mg^{2+}, is advantageous because it is known to be important for Mg^{2+} binding.

The modifications according to the invention which involve the 2'-R substitution of nucleotides in the catalytic/cleavage region of the oligonucleotide can provide a desirable increase in stability against degradation and can increase catalytic

activity due to improved Mg^{2+} binding.

Replacement of the -G-G-A-G-C-C-C-C- loop sequence or the -C-C-G-U-U-A-G-G- loop sequence with aliphatic diol bridges simplifies the large scale synthesis of these oligonucleotides and eliminates unwanted intermolecular interactions. Oligonucleotides according to the invention with diol bridges can be made by machine more easily and more cheaply than conventional ribozymes.

The modifications according to the invention which involve substituting diols in the loop region of the oligonucleotide can provide a desirable facilitation of cellular uptake.

The oligonucleotides of the present invention may be used as intermediates for further modification to improve their ease of up-take by the cell (in comparison to unmodified oligonucleotides of the invention and known ribozymes), for example by the attachment of carrier molecules. The embodiments having diol bridges are considered to be particularly useful as intermediates for this purpose.

The oligonucleotides of the present invention are potential antagonists of a wide range of therapeutic targets which involve over-expression of products. By binding to specific targets on mRNA and cleaving the mRNA they can stop translation and hence "switch off" a specific gene. In cases where expression of a product (e.g. an enzyme or a protein) by a gene is causative of an illness or disfunction this could lead to a cure. Alternatively a putative gene as a source of a problem phenomenon could be "switched off" selectively and the therapeutic effect observed. The oligonucleotides of the present invention are potential anticancer and antiviral agents and could also be used as anti-inflammatory and anti-ulcer drugs. The stability of the oligonucleotides of the present invention suggests that they could be used in nanomolar amounts. This offers a significant improvement over known ribozymes which have to be used in relatively large amounts to compensate for their intracellular degradation by nucleases. The

oligonucleotides of the invention may achieve true catalytic activity: i.e. they will not be destroyed in the cleavage reaction.

The invention can also provide novel intermediates which are 2' substituted nucleosides needed to construct the oligonucleotides of the present invention. Such reactive intermediates may be protected where appropriate by a removable protecting group.

The invention can also provide a method which comprises the synthesis of an oligonucleotides according to the invention from building blocks including deoxynucleotides and 2' substituted nucleosides.

Figures

Figure 1 shows the nucleotide sequence of a hammerhead ribozyme.

Figure 2 shows an example of therapeutic target interaction between the myc oncogene mRNA and an oligonucleotide of the invention of the type described in Example 1.

Figure 3 shows a synthetic scheme for producing 2' - CF_2 H substituted nucleosides.

Figure 4 shows a synthetic scheme for producing 2' - COOH substituted nucleosides.

Figure 5 shows a synthetic scheme for producing the nucleoside 9-(2'-C-difluoromethyl - ß - D arabino - furanosyl) - 6 Ethoxy purine.

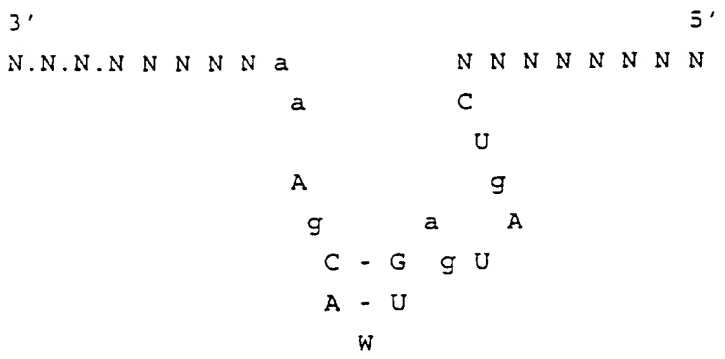
Examples

The following are given as examples of the present invention.

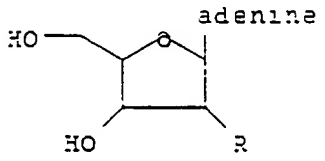
Example 1

An oligonucleotide having the following sequence

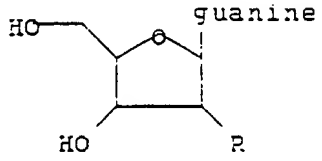
9



in which
N represents any deoxyribonucleotide recognition sequence specific for the target RNA
N.N represents a thiophosphodiester linkage replacing a natural 3'-5' phosphodiester linkage between two nucleotides
A is deoxyadenosine
C is deoxycytidine
G is deoxyguanosine
U is deoxyuridine
a is 2'substituted deoxyadenosine according to formula Ia below
g is 2'substituted deoxyguanosine according to formula Ib below



Ia



Ib

in which R is selected from
$-CF_2H$; -COOH ; $-CONH_2$; $-NHCOR_1$; - SH ; and combinations thereof; R_1 being -H, -C(O)OH or $-CH_2$-C(O)OH

and

W is nucleotide loop sequence IIa.

```
3'                  5'
 -G-G-A-G-C-C-C-C-              IIa
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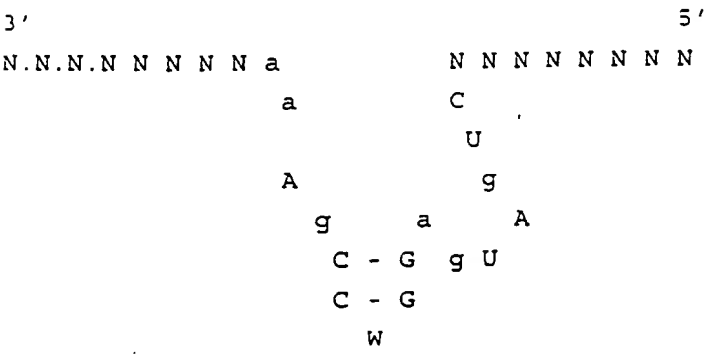
Example 2

An oligonucleotide having the following sequence



in which

N represents any deoxyribonucleotide recognition sequence specific for the target RNA

N.N represents a thiophosphodiester linkage replacing a natural 3'-5' phosphodiester linkage between two nucleotides

A is deoxyadenosine

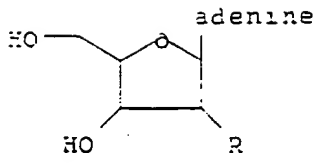
C is deoxycytidine

G is deoxyguanosine

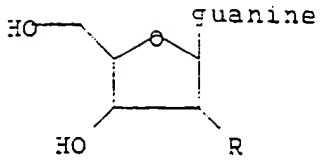
U is deoxyuridine

a is 2'substituted deoxyadenosine according to formula Ia below

g is 2'substituted deoxyguanosine according to formula Ib below



Ia



Ib

in which R is selected from
$-CF_2H$; -COOH ; $-CONH_2$; $-NHCOR_1$; - SH ; and combinations thereof; R_1 being -H, $-C(O)OH$ or $-CH_2-C(O)OH$
and
W is nucleotide loop sequence IIb.

3′ 5′
-C-C-G-U-U-A-G-G- IIb

Example 3

An oligonucleotide having the following sequence

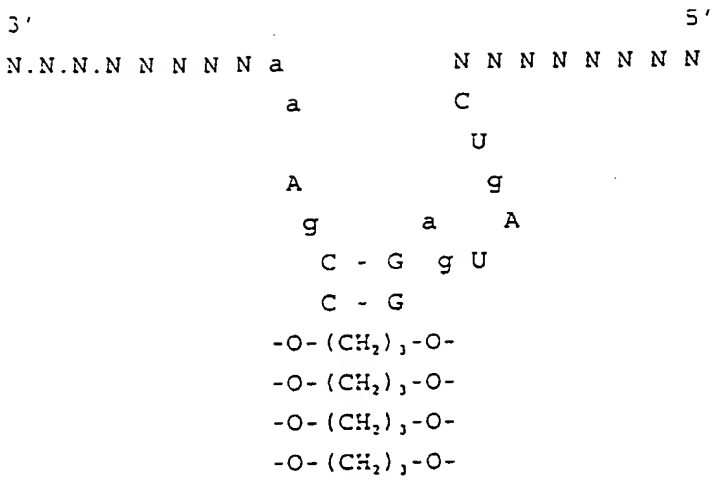
3′ 5′
N.N.N.N N N N N a N N N N N N N N
a C
U
A g
g a A
C - G g U
A - U
$-O-(CH_2)_3-O-$
$-O-(CH_2)_3-O-$
$-O-(CH_2)_3-O-$
$-O-(CH_2)_3-O-$

in which N, N.N, A, C, G, U, a and g are as defined for Example 1 and the diol bridges are connected with phosphodiester or substituted neutral phosphotriester derivative linkages.

Example 4

An oligonucleotide having the following sequence

12



in which N, N.N, A, C, G, U, a and g are as defined for Example 1 and the diol bridges are connected with phosphodiester or substituted neutral phosphotriester derivative linkages.

Example 5

A therapeutic target interaction between mRNA and an oligonucleotide of the invention is shown in Figure 2. The -myc oncogene mRNA secondary structure is shown from nucleotide 1 to nucleotide 900. Translation starts at nucleotide 421 and the triplet cleavage site is positions 433 to 435 (GUU).

Example 6

A schematic synthesis for 2' -CF_2H substituted nucleosides is shown in Figure 3.

B is a purine or pyrimidine heterocycle, i.e. adenine, cytosine, guanine or uracil which may be protected where appropriate.

Compound 7 wherein B is 6 - Ethoxypurine is 9 - (2'- C difluoromethyl D - arabinofuranosyl)

- 6 - Ethoxypurine

Example 7

A schematic synthesis for 2' - COOH substituted nucleosides is

shown in Figure 4.

B is as given in Example 6.

Compound 14 wherein B is Uridine is 3-(2' - C - Carboxyl-Uridine D - arabino furanosyl) - Uridine

Example 8

Synthesis of 2'-Difluoromethyl Substituted Nucleoside

One group of novel analogs are 2'-difluoromethyl substituted nucleoside analogs. Below is described the synthesis for a representative analog corresponding to Compound 7 of Example 6. The synthetic scheme is shown in Figure 5.

6-Ethoxy-N9-ß-D-ribofuranosylpurine was prepared according to literature procedures from inosine.

6-Ethoxy-3'-5'-O-(1,1,3,3-tetraisopropyldisiloxyl)-N9-ß-D-ribofuranosylpurine(1)
To a solution of 1 (1.67 g, 5.64 mmol) in pyridine (50 ml) $TIPDSiCl_2$ (1.94 ml, 6.20 mmol) was added. The mixture was stirred at rt for 3 hrs, evaporated and coevaporated with toluene (20 ml). The oily residue was partitioned between water (50 ml) and diethylether (150 ml). Combined organic layers were dried with sodium sulfate, filtered and evaporated to give a yellowish oil (2.98 g, 98%). FCC (5 cm x 15 cm, petrol ether/ethylacetate 2:1) yielded 2.37 g (78%) of a colorless oil.

1H-NMR: 8.45 (s, 1H), 8.07 (s, 1H), 6.02 (d, 1H), 5.07 (m, 1H), 4.64 (q, 2H), 4.54 (d, 1H), 4.10 (m, 3H), 3.24 (s, 1H), 1.50 (t, 3H), 1. 1 0 (m, 28H).

6-Ethoxy-3',5'O-(1,1,3,3-tetraisopropyldisiloxyl)-N9-b-D-ribofuran-2-ulosylpurine (2)
To a suspension of chromium(VI)oxide (0.400g, 4 mmol) in dichloromethane (12 ml) pyridine (0.644-mi, 8 mmol) was added and the deep red solution was stirred at rt for 10'. Subsequent

addition of 2 (0.539 g, 1 mmol) and acetic anhydride (0.38 ml, 4 mmol) gave a brownish suspension. After stirring at rt for 1 hr the mixture was added to a supernatant of ethylacetate (80 ml) over a 3 cm layer of silica gel in a 3 cm column. After washing the column with additional 50 ml of ethylacetate the filtrate was evaporated (waterbath below 25°C!). Subsequent coevaporation with toluene, chloroform and diethylether (30 ml each) yielded a colorless oil (0.054 g, 94%). 3 was directly used for the synthesis of 4.

6-Ethoxy-2'-{1,1-difluoro-1-(phenylsulfonyl)methyl}-3',5'-O-(1,1,3,3-tetraisopropyldisiloxyl)-N9-ß-D-arabinofuranosylpurine(3)

To a solution of 3 (0.404 g, 0.75 mmol) in THF (10 ml) and HMPA (1 ml) difluoromethylphenylsulfone (1 mmol, 0.192 g) was added and the mixture was cooled to -78°C. LDA (1 ml of a 1.5 M solution in THF, Aldrich) was added and the reaction mixture was stirred at this temperature. TLC after 1 hr indicates complete conversion. After warming to rt saturated aqueous ammonium chloride (20 ml) was added and the mixture was extracted with chloroform (100 ml). Combined organic layers were dried with sodium sulfate, filtered and evaporated to yield 1.32 g of a brownish oil. FCC (3 cm x 15 cm, dichloromethane/methanol 97.3) afforded 4 as a yellowish foam (0.33 g, 61%). 1 H-NMR: 8.41 (s, 1H), 8.18 (s, 1H), 7.84 (m, 2H), 7.73 (m, 1H), 7.54 (m, 2H), 6.73 (s, 1H), 6.29 (s, 1H), 4.99 (d, 1H), 4.67 (q, 2H), 4.05 (dd, 2H), 3.98 (m, 1H), 1.49 (t, 3H), 1.15 (m, 28H).

6-Ethoxy-2'-C-difluoromethyl-N9-ß-D-arabinofuranosylpurine (7)

To a mixture of 4 (0.218 g, 0.3 mmol) and a 0.1 M solution of SmI_2 in THF (1 5 ml, 1.5 mmol) HMPA (0. 60 ml, 3.45 mmol) was added. The color changed from deep blue to purple. The mixture was stirred at rt. The purple color changed gradually to greenish within 10' after which TLC indicated complete reaction 2 ml of saturated aqueous ammonium chloride were added and the resulting mixture was evaporated. The residue was partitioned between saturated sodium thiosulfate (40 ml) and ethylacetate (50 ml). Combined organic layers were dried with

sodium sulfate, filtered and evaporated to yield 0.187 g (106%) of a brownish foam. The crude 4 (homogeneous on tlc) was dissolved in THF (10 ml) and TBAF (0. 1 M in THF, Aldrich, 0.6 ml, 0.66 mmol) was added. The yellowish solution was stirred at rt overnight. After evaporation the residue was purified by FCC (2 cm x 12 cm, chloroform/methanol 9:1) to give a colorless amorphous solid (70 mg, 67%).
^{1}H-NMR: 8.52 (s, 1H), 8.42 (s, 1H), 6.46 (m, 2H), 6.23 (t, 1H, J = 50Hz), 6.14 (d, 1H), 5.21 (m, 1H), 4.58 (m, 2H), 4.36 (m, 1H), 3.86 (m, 1H), 3.67 (m, 2H), 1.41 (t, 3H).

^{19}F-NMR spectra of compounds 3 and 7 are in total agreement with the above structures.

6-Ethoxypurine is a synthetic synthon for adenine; we did not convert to an adenosine derivative on the nucleoside level, because the 6-ethoxy group is a suitable base protecting group during oligonucleotide synthesis.

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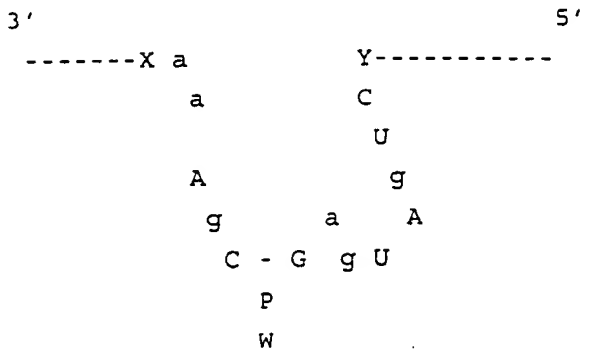
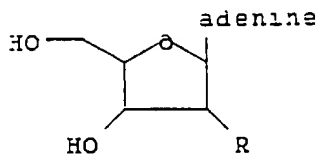
Claims

1. An oligonucleotide having the following sequence

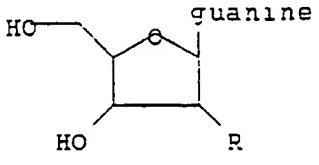


in which

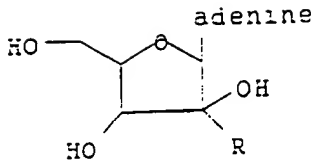
----X and Y---- are target specific RNA recognition sequences
A is deoxyadenosine
C is deoxycytidine
G is deoxyguanosine
U is deoxyuridine
a is 2'substituted deoxyadenosine according to formula Ia below, or 2' substituted arabinofuranosyl adenosine according to formula Ic below or, adenosine
g is 2'substituted deoxyguanosine according to formula Ib below, or a 2' substituted arabinofuranosyl guanosine according to formula Id below, or guanosine



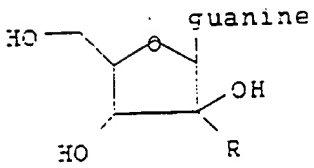
Ia



Ib



Ic



Id

in which R is selected from
$-CF_2H$; -COOH ; $-CONH_2$; $-NHCOR_1$; - SH ; and combinations thereof; R_1 being -H, -C(O)OH or $-(CH_2)C(O)OH$

and in which

and wherein at least one of the groups a or g of the oligonucleotide is other than adenosine or guanosine respectively;

P is either a) A-U or b) C-G

W is either i) nucleotide loop sequence IIa if P is A-U;

3' 5'
-G-G-A-G-C-C-C-C- IIa

or nucleotide loop sequence IIb if P is C-G;

19

3' 5'

-C-C-G-U-U-A-G-G IIb

or ii) diol bridges III connected with phosphodiester or substituted neutral phosphotriester derivative linkages

m (Z) III

in which each diol bridge (Z) is of formula IV

$$- [O - (CH_2)_n - O] - \quad IV$$

n is 1-10 and m diol bridges are connected together, m being 2 to 10.

2. An oligonuceotide according in claim 1 in which there is more than one appearance of a 2' substitution in the sequence.

3. An oligonucleotide according to claim 1 or 2 in which W is diol bridges III and n is 3 in each diol bridge and m is 4.

4. An oligonucloetide according to any preceding claim in which X and Y, which may be the same or different length, are each 4 to 25 nucleotides long.

5. An oligonucleotide according to claim 4 in which X and Y are each 6 to 20 nucleotides long.

6. An oligonucleotide according to any preceding claim in which X or Y include stabilising modifications.

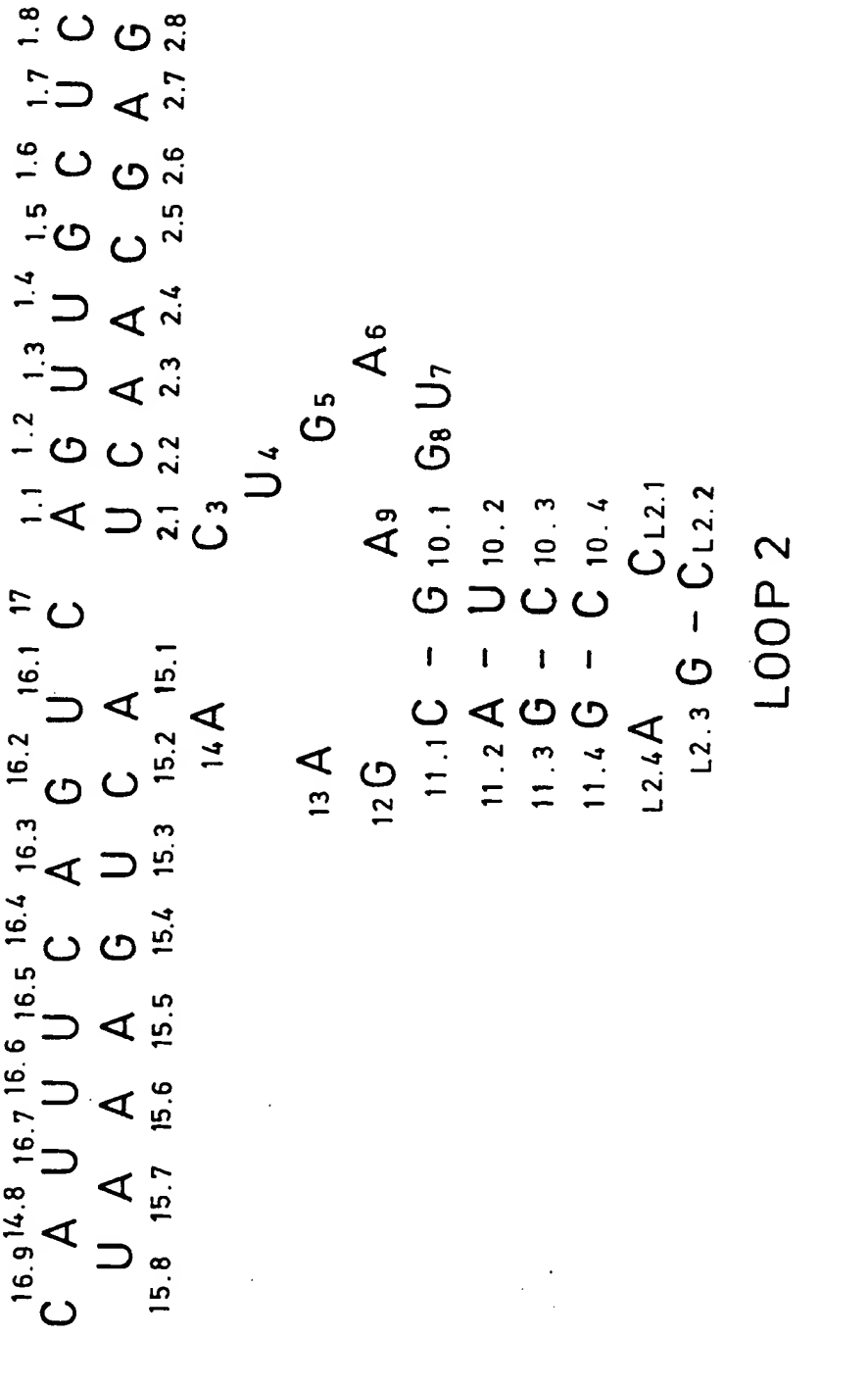
7. An oligonucleotide according to any preceding claim in which three natural 3'-5' phosphodiester- linkages at the 3' end of X are replaced by thiophosphodiester linkages.

8. An oligonucleotide according to any preceding claim, in which W is diol bridges, for use as an intermediate in the preparation of a modified oligonucleotide exhibiting enhanced cellular uptake.

9. A 2' substituted nucleoside a or g as defined in claim 1, or a protected derivative thereof, for use an an intermediate in the preparation of an oligonucleotdie according to claim 1.

10. A method for synthesising an oligonucleotide according to claim 1 which method uses in a synthetic step an intermediate nucleoside according to claim 9.

FIG. 1


HELIX III
HELIX I
LOOP 2
C A U U U C A G U C A G U U G C U C
U A A A G U C A U C A A C G A G
A
G
A
G
C – G
A – U
G – C
G – C
A
G – C

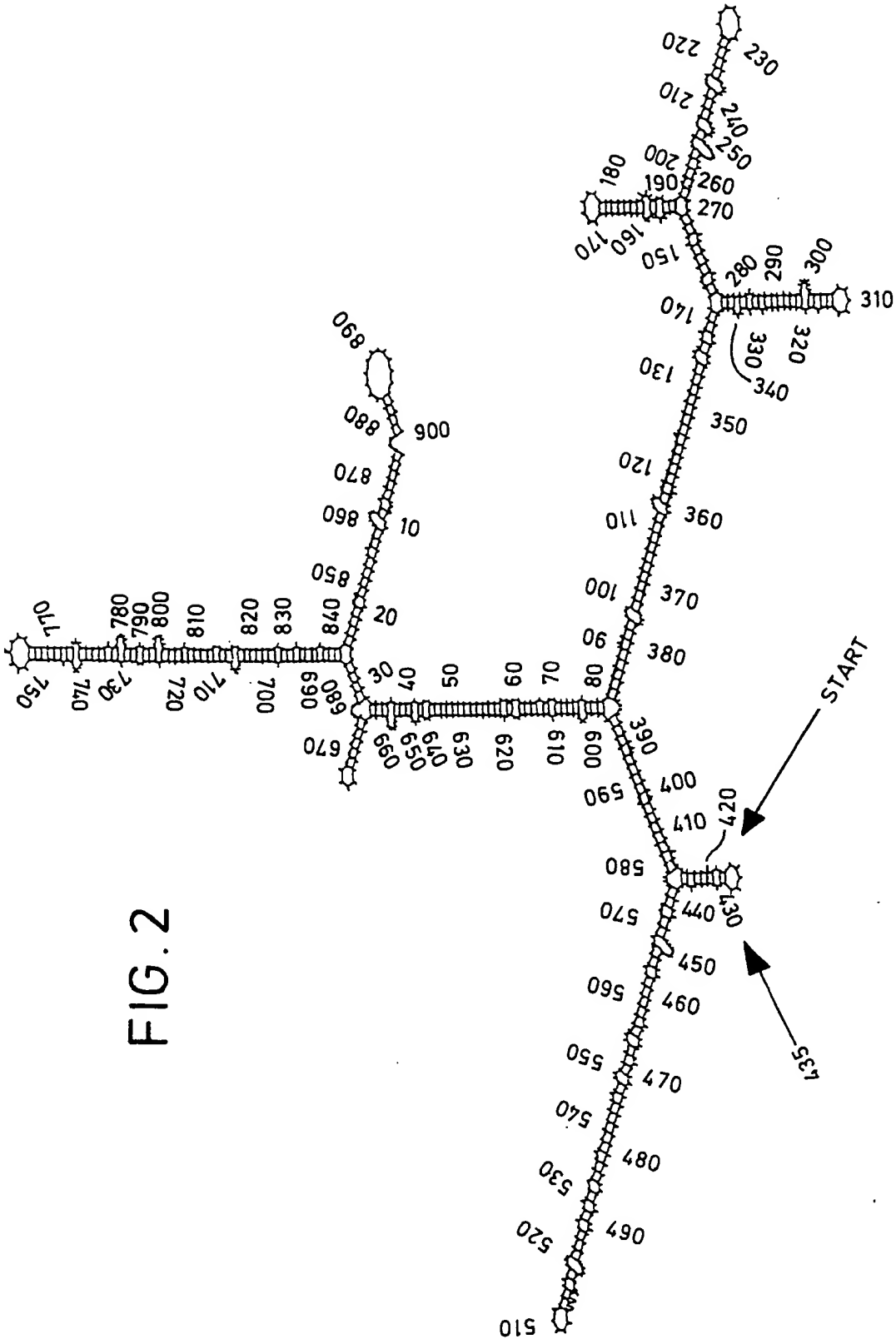


FIG. 2

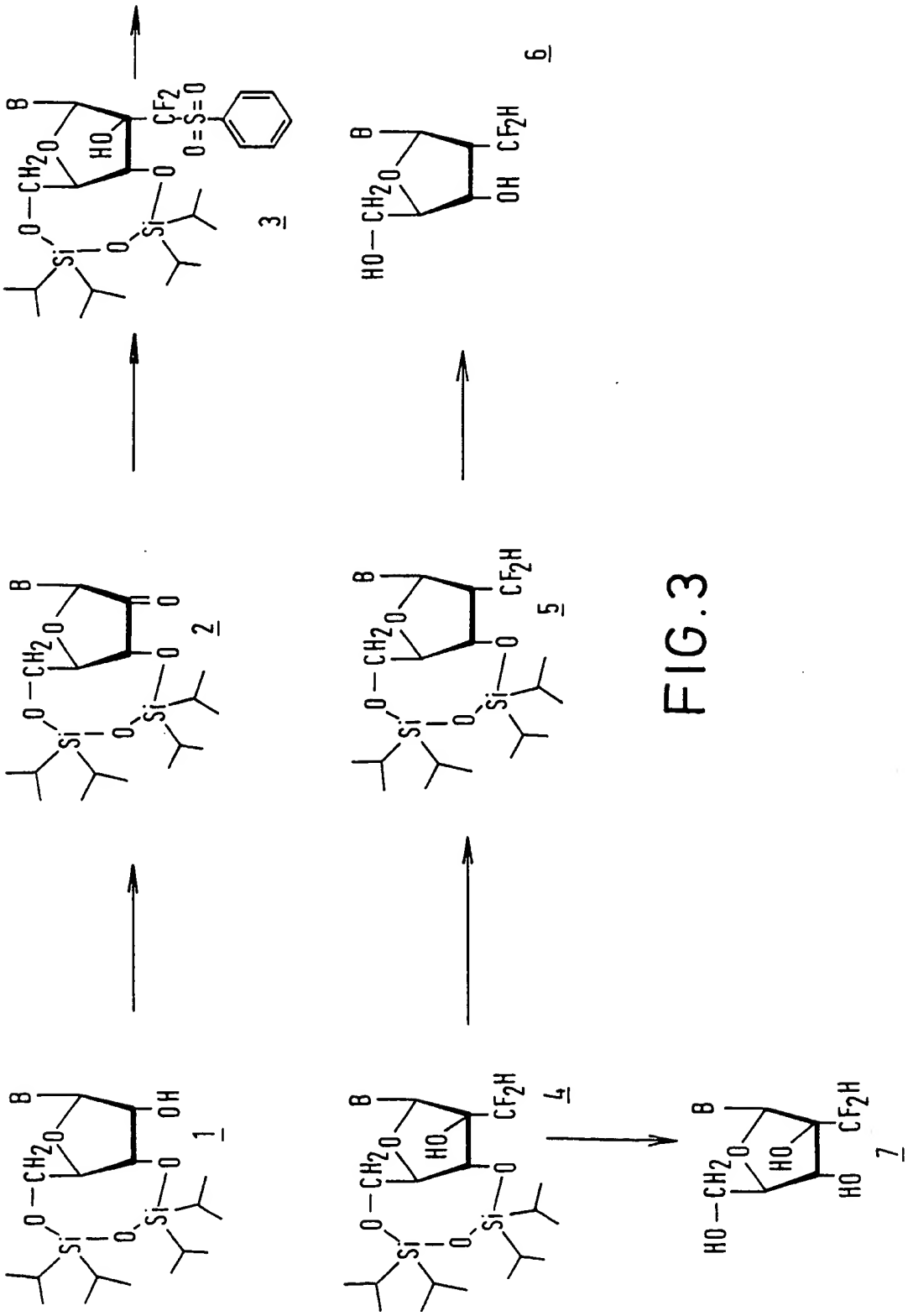


FIG.3

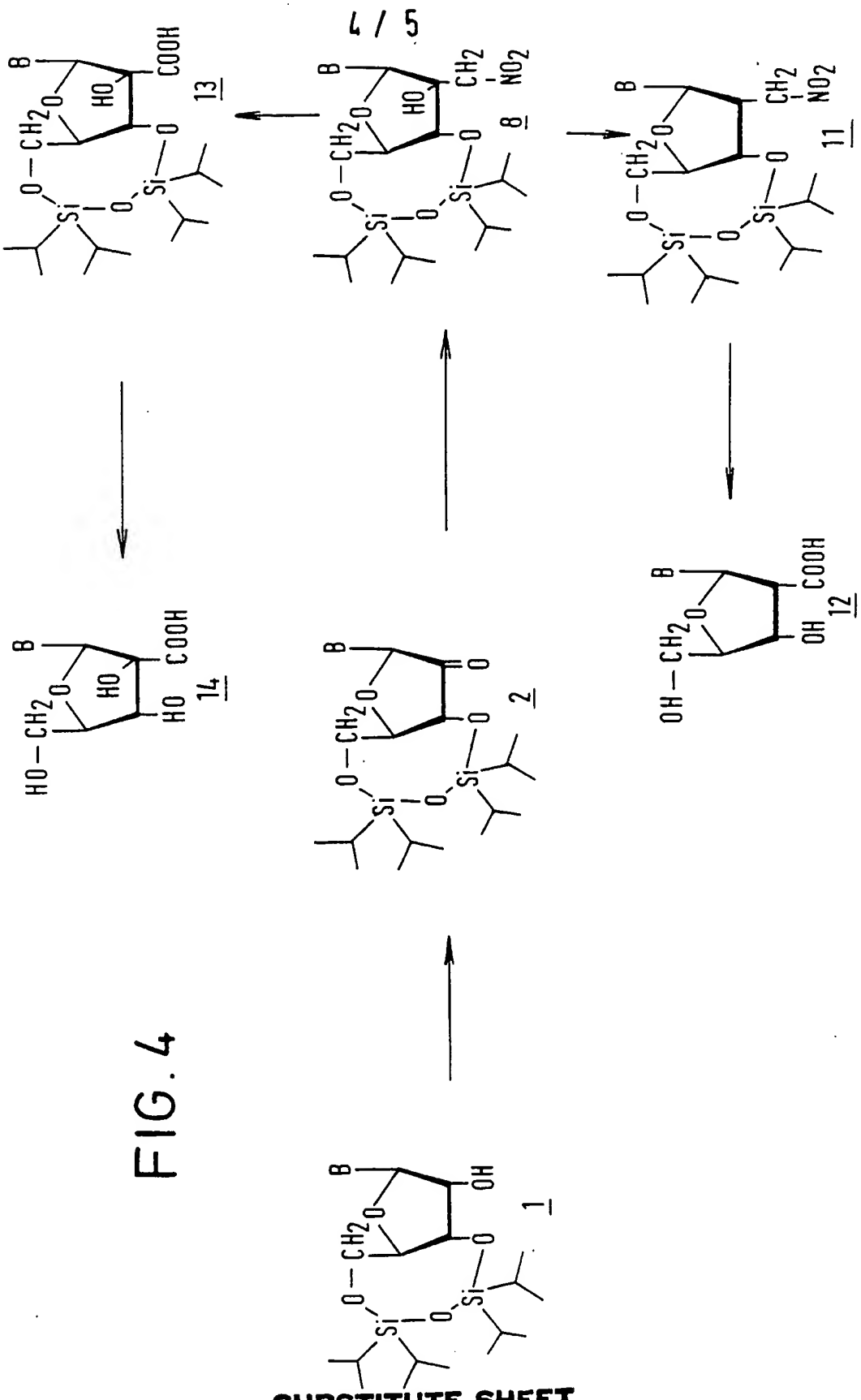


FIG. 4

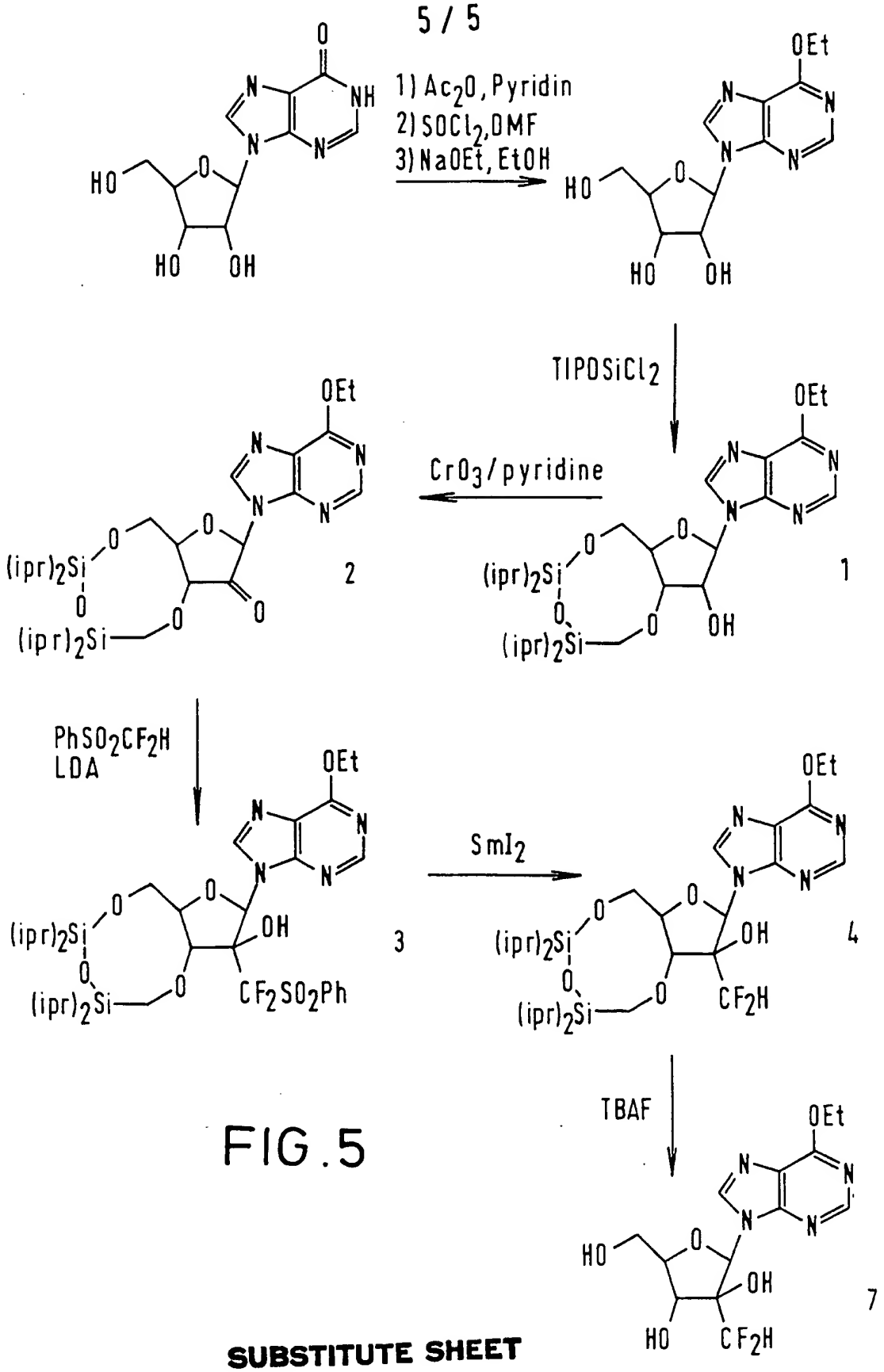

1) Ac_2O, Pyridin
2) $SOCl_2$, DMF
3) NaOEt, EtOH
TIPOSiCl2
CrO_3/pyridine
$PhSO_2CF_2H$
LDA
SmI_2
TBAF
1
2
3
4
7

FIG.5